Supporting Information

The Role of Phosphate in a Multistep Enzymatic Reaction: Reactions of the Substrate and Intermediate in Pieces

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1) Synthesis and Characterization of Truncated and Complete Intermediates

1.1) Alternative Synthetic Route to 2-*C*-methyl-D-glyceraldehyde (2MGA)

(2*R*)-2,3-Dihydroxy-*N*-methoxy-2,*N*-dimethylpropionamide (**3**) was converted to its acetonide using previously described conditions[1] with minor modifications, as follows. Neat **3** was dissolved in 10 equiv of 2,2-dimethoxypropane containing 1 equiv *p*-toluenesulfonic acid. The resulting mixture was stirred for 20 h and partitioned between saturated $NaHCO_3$ and CH_2Cl_2, extracted with CH_2Cl_2, dried over $NaSO_4$, concentrated and purified by flash silica gel chromatography (1:1 EtOAc:hexanes) to produce colorless oil **4** (83 % yield). **4** was reduced in THF for 1 h at 0 °C with 1.25 equiv of $LiAlH_4$, followed by extraction with EtOAc and distillation at reduced pressure (73 °C, 70 mmHg); the distillation conditions were adopted from the protocol for distillation of the (*S*)-glyceraldehyde acetonide.[2] The purified acetonide of 2MGA (**5**) was deprotected in the presence of Amberlite IRA-120 (H^+) in water at room temperature overnight, followed by filtration and concentration, resulting in a colorless oil (30 % yield starting from **4**). The ^{1}H NMR spectrum of the product was in agreement with that described in the Experimental Procedures of the main text.

1.2) Quantification of 2MGA Stock and Determination of Hydration Equilibrium Constant

The concentration of the 2MGA solution was determined through quantitative integration of the ^{1}H NMR signal of its methyl group using gravimetrically determined sodium phosphite as an internal standard. To ensure accurate integrals for the protons of interest, a relaxation delay between pulses of 50 s ($> 8T_1$) was used.[3] The equilibrium constant for hydration (K_{hyd}) of the carbonyl of 2MGA was determined in a solution containing 50 mM sodium phosphite, 25 mM HEPES (adjusted to pH[4] 7.5 with NaOH), and 10 mM $MgCl_2$, at 25 °C upon integration of ^{1}H NMR signals corresponding to the methyl group of its hydrate and aldehyde form. To avoid correction of K_{hyd} for the solvent deuterium isotope effect,[4] a D_2O-filled coaxial insert was used. The concentration of the aldehyde form was determined by multiplying the total concentration of 2MGA by $1/(1 + K_{hyd})$, where $K_{hyd} = 4.9$.

1.3) Determination of the Enantiomeric Purity of 2MGA

Racemic 2MGA was prepared by the protocol described in the Experimental Procedures of the main manuscript, substituting $(DHQ)_2PHAL$ with 0.08 equiv of pyridine during the dihydroxylation step. For the purpose of enantiomeric excess determination, 2MGA and its racemate were converted to their dinitrophenylhydrazones according to the published procedure.[5] *ee* = 94%, determined by HPLC

analysis [Chiralpak AD-RH, 20% iPrOH/hexane, 1.0 mL/min, λ = 345 nm, t_R (major) = 23.65 min, t_R (minor) = 13.59 min].

1.4) Synthesis of 2-*C*-methyl-D-erythrose 4-phosphate (MEsP)

2,3-*O*-Isopropylidene-2-*C*-methyl-D-erythrose 4-dibenzyl phosphate (**8**) was suspended in methanol (50 mg/3 mL) and hydrogenated over 10% Pd/C (6 mg) at room temperature and 40 psi for 30 min. The catalyst was removed by filtration through a 4–5.5 μm sintered glass funnel. The filtrate was diluted with 20 mL of deionized water and concentrated 10 times to remove traces of methanol. The resulting acidic solution of 2,3-*O*-isopropylidene-2-*C*-methyl-D-erythrose 4-phosphate was heated for 9 h at 37 °C, concentrated, and adjusted to pH 7.5 with 1 M NaOH. The ^{1}H NMR spectrum of the product was in agreement with that previously reported.[6]

1.5) Quantification of MEsP Stock and Determination of Hydration Equilibrium Constant

The concentration of the MEsP solution was determined through quantitative integration of ^{1}H NMR signal of its methyl group using gravimetrically determined sodium phosphite as an internal standard. The equilibrium constant for hydration (K_{hyd}) of the carbonyl of MEsP was determined in its 2.7 mM solution in H_2O at 25 and 37 °C upon integration of ^{1}H NMR signals corresponding to the methyl group of its hydrate and aldehyde form. The concentration of the aldehyde form was determined as described for 2MGA using K_{hyd} = 1.4 and 1.0 at 25 °C and 37 °C, respectively.

2) Chemical Competence of Intermediates.

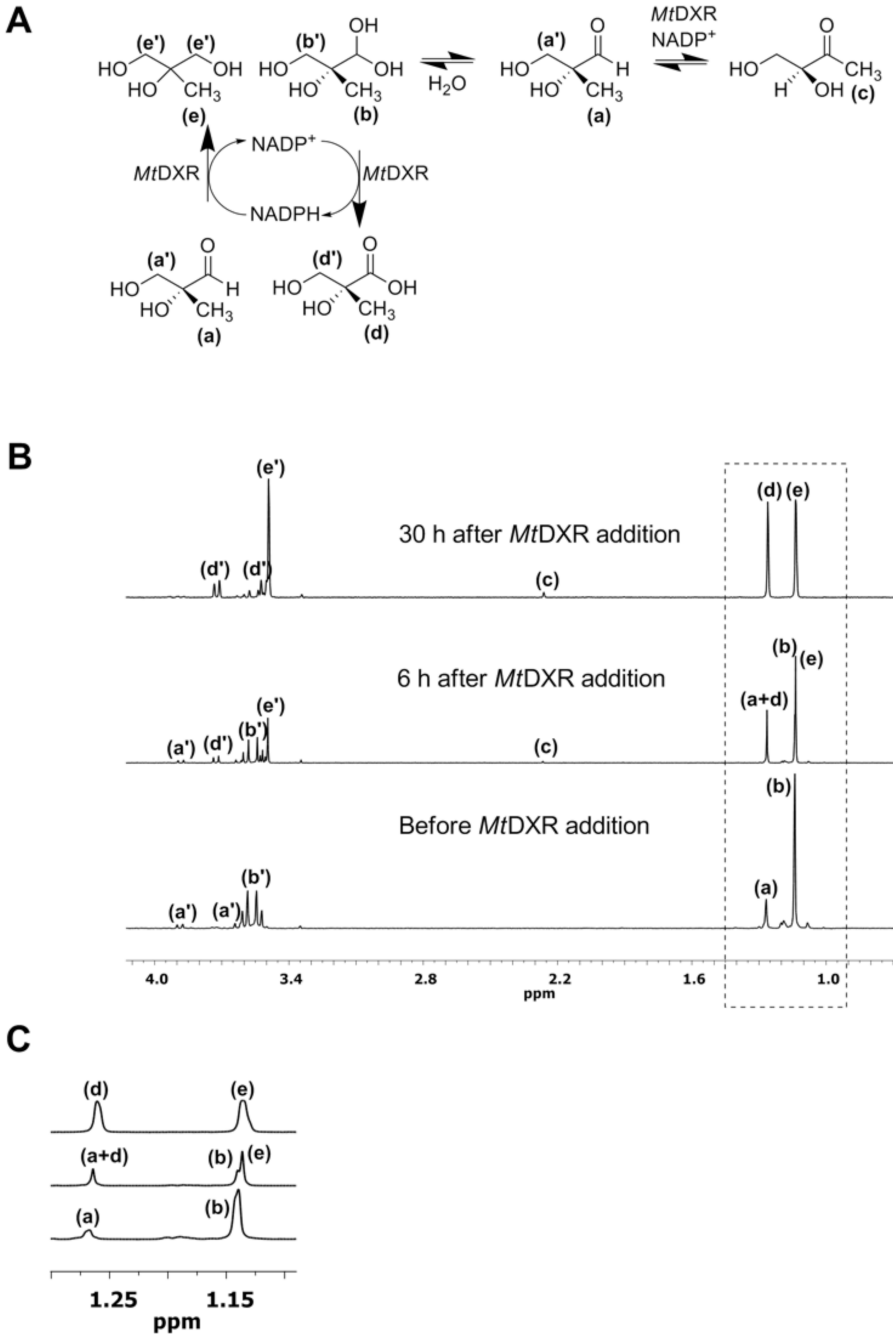


Figure S1. (A) *Mt*DXR-catalyzed partitioning of 2MGA (a) to DE (c) or a mixture of 2-*C*-methyl-D-glyceric acid (d) and 2MG (e) in the presence of $NADP^+$ and phosphite dianion. (B) ^{1}H-NMR spectra of the partitioning of 20 mM 2MGA (aldehyde + hydrate) in the presence of 50 μM *Mt*DXR, 1.6 mM $NADP^+$, 50 mM sodium phosphite buffer, and 10 mM $MgCl_2$ at pH 7.5, 25 °C. (C) Zoomed region depicted by the dashed box in (B).

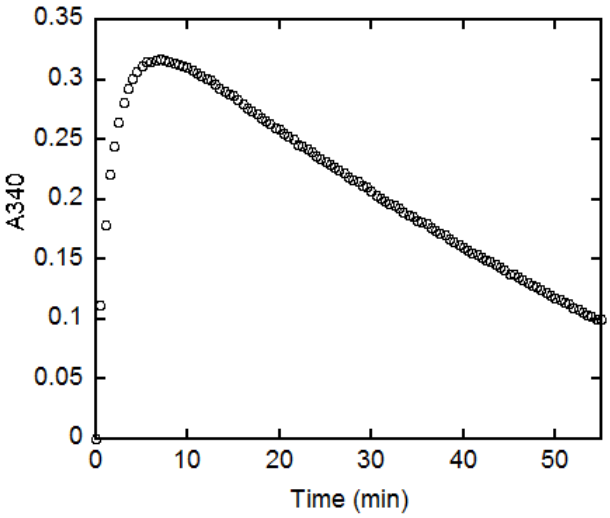


Figure S2. UV spectrophotometric time course of the incubation of 2MGA with $NADP^+$ and *Mt*DXR in the following conditions: 50 µM *Mt*DXR, 1.4 mM $NADP^+$, 17.5 mM 2MGA (aldehyde + hydrate), 50 mM sodium phosphite buffer, 10 mM $MgCl_2$ at pH 7.5, 25 °C.

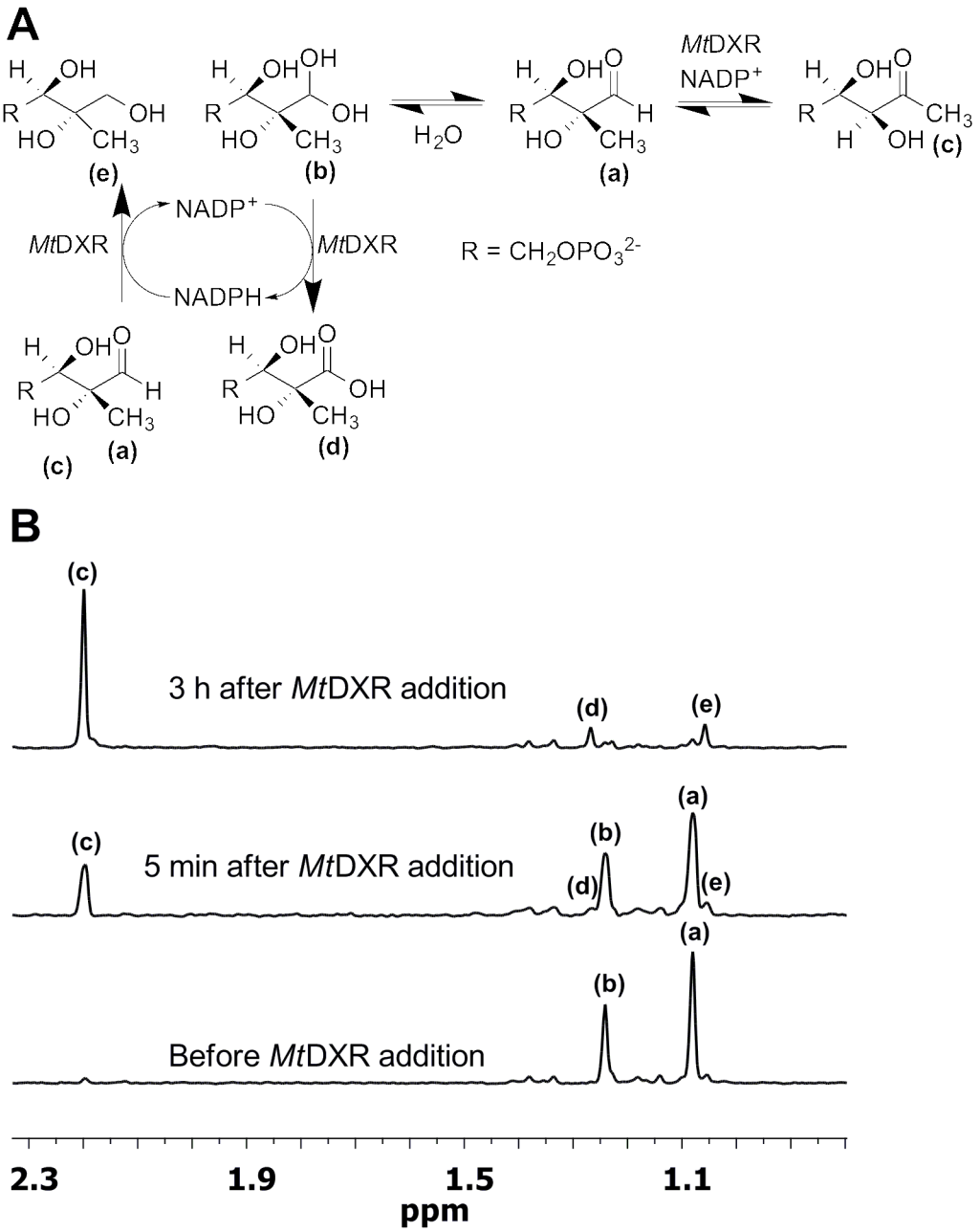


Figure S3. (A) *Mt*DXR-catalyzed partitioning of MEsP (a) to DXP (c) or a mixture of 2-*C*-methylglyceric acid (d) and MEP in the presence of $NADP^+$; (B) ^{1}H-NMR spectra of the abovementioned partitioning in the following conditions: 20 µM *Mt*DXR, 2.5 mM $NADP^+$, 2 mM 2MGA (aldehyde + hydrate), 50 mM sodium phosphite buffer, 10 mM $MgCl_2$ at pH 7.5, 25 °C.

3) Rate-Limiting Steps for Substrates and Intermediates

3.1) Determination of KIEs on the Hydride Transfer Step

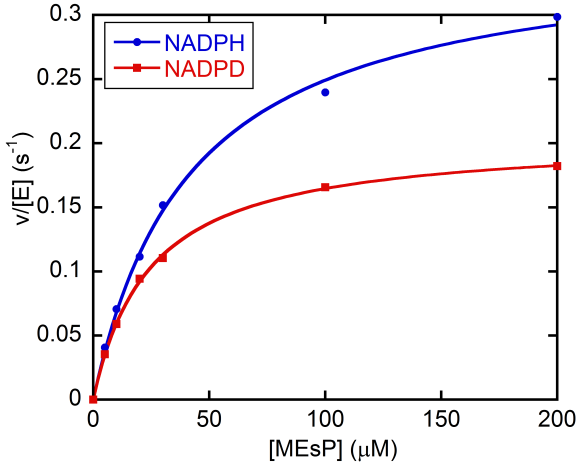


Figure S4. Steady-state kinetics for *Mt*DXR-catalyzed reduction of MEsP with NADPH and NADPD

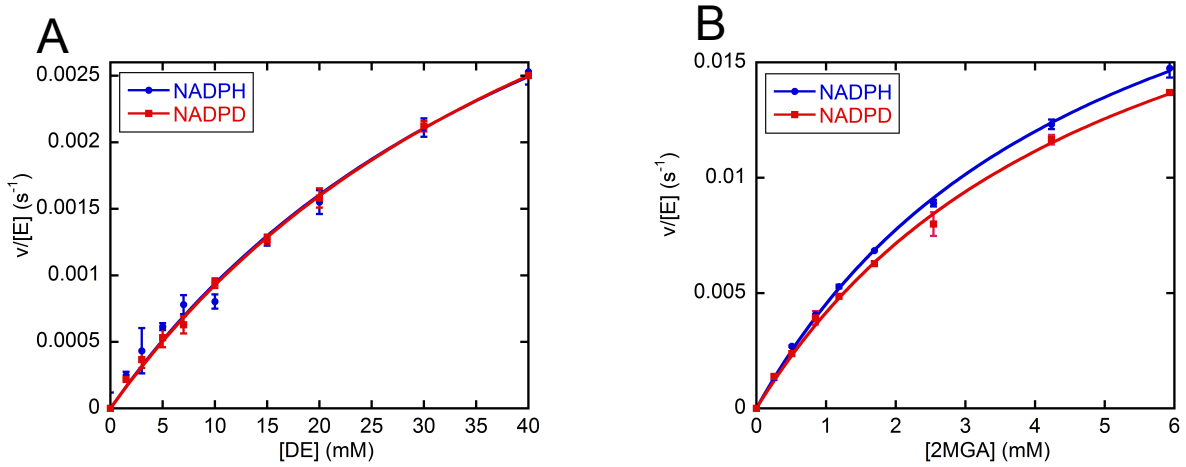
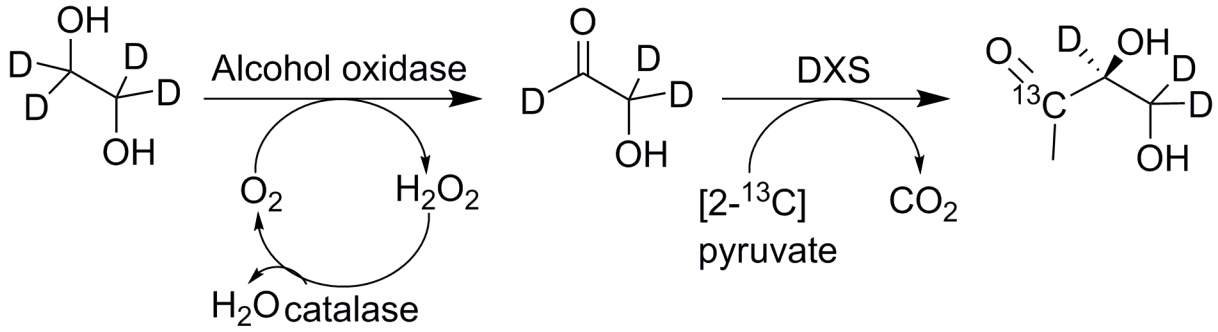


Figure S5. Steady-state kinetics for *Mt*DXR-catalyzed reaction of DE (A) and 2MGA (B) with NADPH and NADPD at $[HPO_3^{2-}]$ = 47 mM. Error bars represent standard deviations from three measurements.

3.2) Determination of KIE on the Isomerization of Substrate in Pieces

Scheme S1. Chemoenzymatic Synthesis of (2-^{13}C;3,4,4-$^{2}H_3$)DE.



Acquisition and Processing of ^{13}C NMR Spectra

The ^{13}C NMR spectra for the 3,4,4-$^{2}H_3$ KIE were acquired at 125 MHz at 25 °C in a standard grade 5-mm diameter NMR tube. The probe was tuned and samples were manually shimmed before and after addition of NADPH using the VnmrJ program package (Version 2.2D). For each spectrum, 4 dummy scans and 300 scans were accumulated with a relaxation delay between scans, τ_{rel}, of 10 s (120 min measurement time per spectrum). One preliminary spectrum was acquired for each sample, and the reaction was then initiated by adding NADPH. The shimming was immediately readjusted, and acquisition commenced within 3 min of the initiation of the reaction. Data acquisition continued until the concentration of the residual substrate reached 15% of its initial concentration (typical experiment time 36 h). Fourier transformation of the FIDs was performed after zero filling to 1024k points and application of a Gaussian function of 1.0 Hz. Manual phasing and automatic baseline correction (Whittaker smoothing) were applied. The line fitting function of the MestReNova program (version 7.1; Mestrelab Research, Spain) was used to the integrate peaks arising from heavy and light isotopologues as well as the glycine peak.

The Lorentzian/Gaussian curve function, peak width, and peak height for each signal were allowed to vary for each signal throughout the experiment. The peak areas were recorded for calculation of F_1 and R.

^{13}C NMR data for the 3,4,4-$^{2}H_3$ KIE were analyzed as follows. For each spectrum, the peak area of the ($^{13}C_1$)DE singlet, i_1, was divided by the peak area of the (1-^{13}C)glycine standard, i_{Std}. The fraction of ($^{13}C_1$)DE (i.e., light isotopologue) converted, F_1, was calculated from eq S1, where "0" and "F" in the subscripts indicate before reaction and after partial conversion, respectively.

$$F_1 = \frac{i_{1,F} / i_{Std,F}}{i_{1,0} / i_{Std,0}} \qquad \textbf{(S1)}$$

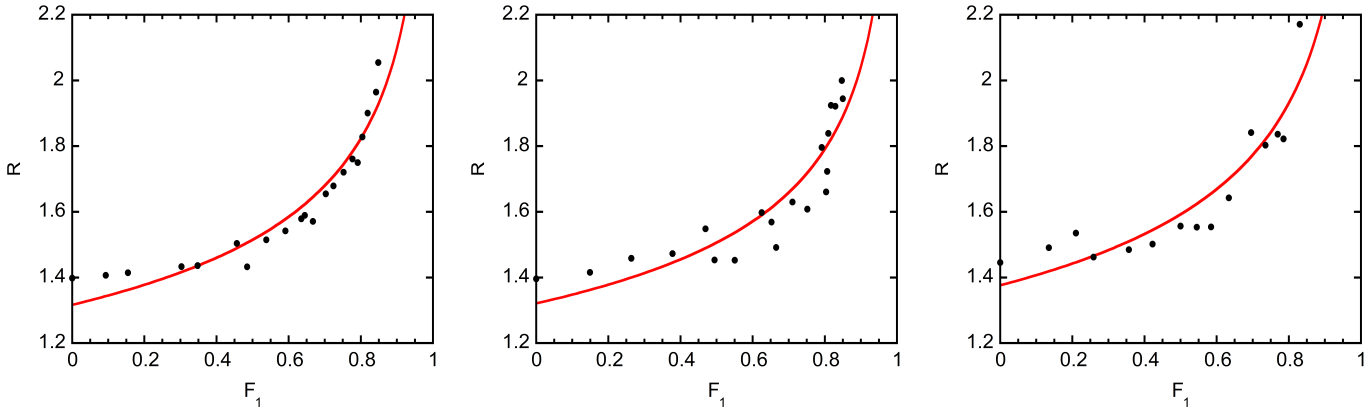
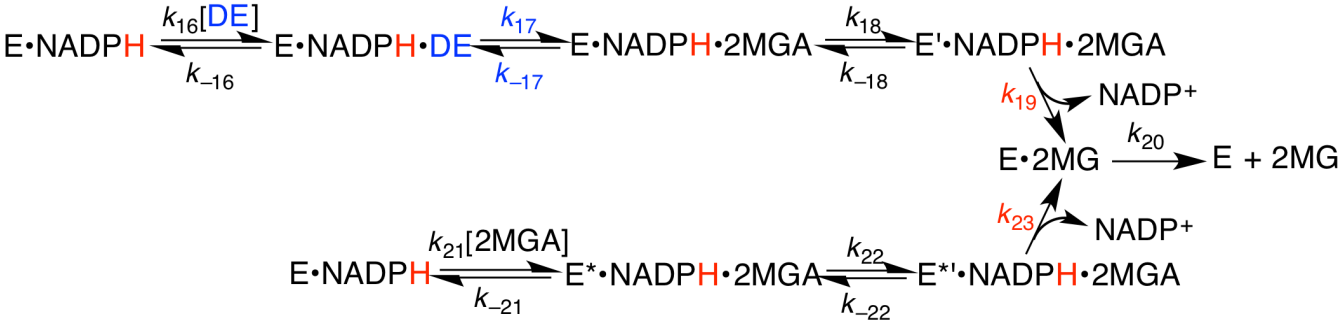


Figure S6. Progress curves for the for the 3,4,4-$^2H_3$ KIE of DE turnover by *Mt*DXR. *R* is the ratio of heavy isotopologue versus light isotopologue and F_1 is the fraction of conversion of the light isotopologue. Fitting of eq 3 to each replicate yielded a mean KIE value of 1.25 ± 0.02.

Scheme S2. Simplified Kinetic Model for Turnover of DE and 2MGA.[a]



[a] The hydride/deuteride and its isotope-sensitive step are in red. Deuterium-labeled DE and its isotope-sensitive step are in blue.

4) Kinetic Barrier Stabilization by Phosphodianion

$$\Delta G_{\mathrm{Pi}} = -RT \ln\left[\frac{\left(k_{\mathrm{cat}}/K_{\mathrm{m}}\right)_{\mathrm{S\text{-}Pi}}}{\left(k_{\mathrm{cat}}/K_{\mathrm{m}}\right)_{\mathrm{S}}}\right] \quad \textbf{(S2)}$$

where S-Pi is DXP or MEsP and S is DE or 2MGA, respectively.

$$\Delta G_{\mathrm{HPi}} = -RT \ln\left[\frac{k'_{\mathrm{cat}}/K_{\mathrm{m}}}{\left(k_{\mathrm{cat}}/K_{\mathrm{m}}\right)K_{\mathrm{HPi}}}\right] \quad \textbf{(S3)}$$

5) Derivation of the Rate Equation for *Mt*DXR Turnover of 2MGA

Scheme 4 describes the turnover of 2MGA by *Mt*DXR (note that it is assumed that NADPH is saturating and therefore bound to all species). It is assumed that binding of phosphite dianion (HP_i) is under rapid equilibrium but binding of 2MGA occurs in the steady state, together with turnover. k_{cat} and k_{cat}' include hydride transfer, release of 2MG and $NADP^+$, and internal physical step(s) such as conformational change(s). Using Cha's approach, the rapid equilibrium segments can be rewritten as X and X′, where $[X] = [E] + [E{\bullet}HP_i]$ and $[X'] = [E{\bullet}2MGA] + [E{\bullet}2MGA{\bullet}HP_i]$ and the fractional concentrations of each enzyme form are given by eqs S4 to S7.

$$f_1 = \frac{[\mathrm{E}]}{[\mathrm{X}]} = \frac{K_{\mathrm{HPi}}}{K_{\mathrm{HPi}} + [\mathrm{HP_i}]} \quad \textbf{(S4)}$$

$$f_2 = \frac{[\mathrm{E{\bullet}HP_i}]}{[\mathrm{X}]} = \frac{[\mathrm{HP_i}]}{K_{\mathrm{HPi}} + [\mathrm{HP_i}]} \quad \textbf{(S5)}$$

$$f_{\mathrm{cat}} = \frac{[\mathrm{ES}]}{[\mathrm{X'}]} = \frac{K_{\mathrm{HPi,S}}}{K_{\mathrm{HPi,S}} + [\mathrm{HP_i}]} \quad \textbf{(S6)}$$

$$f'_{\mathrm{cat}} = \frac{[\mathrm{E{\bullet}S{\bullet}HP_i}]}{[\mathrm{X'}]} = \frac{[\mathrm{HP_i}]}{K_{\mathrm{HPi,S}} + [\mathrm{HP_i}]} \quad \textbf{(S7)}$$

The King-Altman method gives eqs S8 and S9.

$$\frac{[\mathrm{X}]}{[\mathrm{E}]_\mathrm{t}} = \frac{\left(k_{-1}+k_{\mathrm{cat}}\right)f_{\mathrm{cat}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)f'_{\mathrm{cat}}}{\left(k_{-1}+k_{\mathrm{cat}}\right)f_{\mathrm{cat}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)f'_{\mathrm{cat}}+\left(k_1 f_1+k_2 f_2\right)[\mathrm{S}]} \tag{S8}$$

$$\frac{[\mathrm{X}']}{[\mathrm{E}]_\mathrm{t}} = \frac{\left(k_1 f_1+k_2 f_2\right)[\mathrm{S}]}{\left(k_{-1}+k_{\mathrm{cat}}\right)f_{\mathrm{cat}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)f'_{\mathrm{cat}}+\left(k_1 f_1+k_2 f_2\right)[\mathrm{S}]} \tag{S9}$$

The rate equation is given by eq S10, which upon substitution with eqs S4 to S9 gives eq S11.

$$v_0 = k_{\mathrm{cat}}[\mathrm{ES}]+k'_{\mathrm{cat}}[\mathrm{E{\bullet}S{\bullet}HP_i}] = \left(k_{\mathrm{cat}}f_{\mathrm{cat}}+k'_{\mathrm{cat}}f'_{\mathrm{cat}}\right)[\mathrm{X}'] \tag{S10}$$

$$\begin{aligned}
\frac{v_0}{[\mathrm{E}]_\mathrm{t}} &= \frac{\left(k_{\mathrm{cat}}f_{\mathrm{cat}}+k'_{\mathrm{cat}}f'_{\mathrm{cat}}\right)\left(k_1 f_1+k_2 f_2\right)[\mathrm{S}]}{\left(k_{-1}+k_{\mathrm{cat}}\right)f_{\mathrm{cat}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)f'_{\mathrm{cat}}+\left(k_1 f_1+k_2 f_2\right)[\mathrm{S}]} \\
&= \frac{\left(k_{\mathrm{cat}}\dfrac{K_{\mathrm{HPi,S}}}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]}+k'_{\mathrm{cat}}\dfrac{[\mathrm{HP_i}]}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]}\right)\left(k_1\dfrac{K_{\mathrm{HPi}}}{K_{\mathrm{HPi}}+[\mathrm{HP_i}]}+k_2\dfrac{[\mathrm{HP_i}]}{K_{\mathrm{HPi}}+[\mathrm{HP_i}]}\right)[\mathrm{S}]}{\left(k_{-1}+k_{\mathrm{cat}}\right)\dfrac{K_{\mathrm{HPi,S}}}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]}+\left(k_{-2}+k'_{\mathrm{cat}}\right)\dfrac{[\mathrm{HP_i}]}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]}+\left(k_1\dfrac{K_{\mathrm{HPi}}}{K_{\mathrm{HPi}}+[\mathrm{HP_i}]}+k_2\dfrac{[\mathrm{HP_i}]}{K_{\mathrm{HPi}}+[\mathrm{HP_i}]}\right)[\mathrm{S}]} \\
&= \frac{\left(k_{\mathrm{cat}}K_{\mathrm{HPi,S}}+k'_{\mathrm{cat}}[\mathrm{HP_i}]\right)\left(k_1K_{\mathrm{HPi}}+k_2[\mathrm{HP_i}]\right)[\mathrm{S}]}{\left(k_{-1}+k_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)K_{\mathrm{HPi,S}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)[\mathrm{HP_i}]+\left(k_1K_{\mathrm{HPi}}+k_2[\mathrm{HP_i}]\right)\left(K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]\right)[\mathrm{S}]} \\
&= \frac{\dfrac{k_{\mathrm{cat}}K_{\mathrm{HPi,S}}+k'_{\mathrm{cat}}[\mathrm{HP_i}]}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]}[\mathrm{S}]}{\dfrac{\left(k_{-1}+k_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)K_{\mathrm{HPi,S}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)[\mathrm{HP_i}]}{\left(k_1K_{\mathrm{HPi}}+k_2[\mathrm{HP_i}]\right)\left(K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]\right)}+[\mathrm{S}]}
\end{aligned} \tag{S11}$$

The apparent k_{cat} and K_m are therefore given by eqs S12 and S13, respectively.

$$k_{\mathrm{cat,app}} = \frac{k_{\mathrm{cat}}K_{\mathrm{HPi,S}}+k'_{\mathrm{cat}}[\mathrm{HP_i}]}{K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]} \tag{S12}$$

$$K_{\mathrm{m,app}} = \frac{\left(k_{-1}+k_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)K_{\mathrm{HPi,S}}+\left(k_{-2}+k'_{\mathrm{cat}}\right)\left(K_{\mathrm{HPi}}+[\mathrm{HP_i}]\right)[\mathrm{HP_i}]}{\left(k_1K_{\mathrm{HPi}}+k_2[\mathrm{HP_i}]\right)\left(K_{\mathrm{HPi,S}}+[\mathrm{HP_i}]\right)} \tag{S13}$$

With the assumption that the association rate constant for 2MGA is the same for both complexes (i.e., $k_1 = k_2$), $K_{m,app}$ simplifies to eq S14, where K_m and K_m' are the Michaelis constant for 2MGA in the absence and presence of phosphite dianion, respectively.

$$\begin{aligned} K_{m,app} &= \frac{k_{-1} + k_{cat}}{k_1} \frac{K_{HPi,S}}{K_{HPi,S} + [HP_i]} + \frac{k_{-2} + k'_{cat}}{k_2} \frac{[HP_i]}{K_{HPi,S} + [HP_i]} \\ &= K_m \frac{K_{HPi,S}}{K_{HPi,S} + [HP_i]} + K'_m \frac{[HP_i]}{K_{HPi,S} + [HP_i]} \end{aligned} \quad \textbf{(S14)}$$

Because $K_{m,app}$ was observed to be independent of $[HP_i]$, K_m equals K_m', and therefore $k_{-1} + k_{cat} = k_{-2} + k_{cat}'$.

6) Determination of the Structure of *Mt*DXR with the Substrate in Pieces

Crystals of *Mt*DXR containing an N-terminal histidine tag were grown at 20 °C by the hanging drop vapor diffusion method with a mother liquor containing 25% PEG 3350, 25 mM sodium acetate, 25 mM Bis-Tris (pH 5.5), 1% Tacsimate (Hampton Research), and 5% 2-methyl-2,4-pentanediol. The *Mt*DXR protein stock (final concentration: 2 mg/mL) was buffered by a solution of 1 mM $MnCl_2$, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% glycerol. The crystallization conditions also contained final concentrations of 40 mM DE, 1 mM NADPH, and 10 mM Na_2HPO_3. Crystals were cryoprotected for data collection by harvesting in a nylon loop that was then passed through a solution of Paratone-N. Data were collected at SSRL beamline 7-1 and indexed in a monoclinic space group. The structure was solved by molecular replacement with PHENIX using 4OOE as a search model. All ligands and heteroatoms were removed prior to molecular replacement. Molecular replacement found two chains in the asymmetric unit. After initial refinement and manual model building, electron density for the ligands allowed inclusion into the molecular model (Figure S7). Data collection and refinement statistics are listed in Table S1.

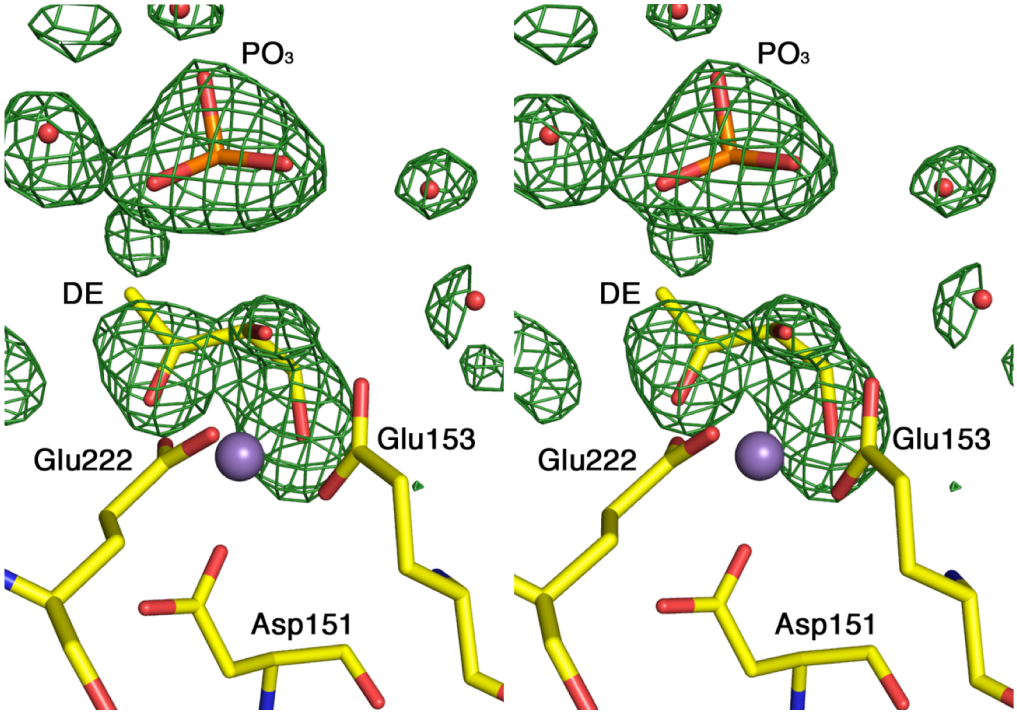


Figure S7. Stereoview of electron density for ligands of *Mt*DXR. Electron density was generated with coefficients of the form Fo-Fc calculated prior to inclusion of DE or HPO_3^{2-} into the molecular model. The density is contoured at 2.5σ. The Mn^{2+} ion is shown in purple and the three protein residues that coordinate the ion are labeled. Several water molecules that were subsequently built into the model are also shown.

Table S1. Diffraction and refinement statistics for *Mt*DXR complex with NADPH, Mn^{2+}, DE, and HPO_3^{2-}

	*Mt*DXR + DE + NADPH + Mn^{2+} + HPO_3^{2-}
PDB Code	4RCV
Beamline	SSRL 7.1
Wavelength	1.1271
Space group	$P2_1$
Unit cell a, b, c (Å)	a=67.37 b=64.38 c=85.74
	α=90.0 β=101.73 γ=90.0
Molecules per asymmetric unit	2
Resolution range (Å)	35.1 – 2.29
No. observations	112891
No. unique reflections	32321
Multiplicity	3.5
Completeness (%)	99.8
I/σ^a	7.6 (1.7)
$R_{merge}{}^a$ (%)	9.7 (47.8)
Structure Refinement	
$R_{factor}{}^a$ (%)	17.3 (20.5)
$R_{free}{}^a$ (%)	23.1 (28.9)
No. protein/water atoms	5446 / 133
RMSD bond distances (Å)	0.010
RMSD bond angles (°)	1.15
Wilson B-factor ($Å^2$)	30.0
Average B-Factor ($Å^2$)	
Protein (all, main, side)	27.0, 26.2, 28.0
Ligands	34.1
Solvent	27.3
Ramachandran analysis (%)	
Favored	724
Outliers	4
Molprobity ClashScore	3.3 (100^{th} percentile)

[a]Values in parentheses represent the statistics within the highest-resolution shell.

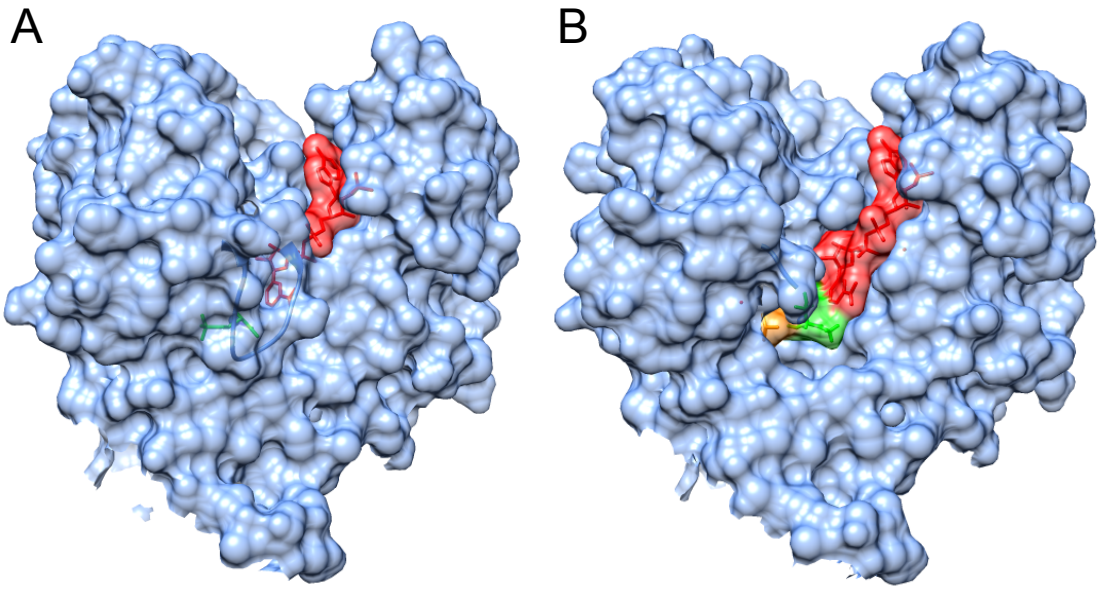


Figure S8. Surfaces of the *Mt*DXR subunit (A) with FR-900098, Mn^{2+} and NADPH (red) bound (PDB entry 4A03) and (B) with DE (green), Mn^{2+}, NADPH (red) and HPO_3^{2-} (orange) bound (PDB entry 4RCV). The electron density for the flexible loop 189–206 is absent in (B), allowing a view of the contents of the active site that are partially obscured in (A).

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